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Tromantadine: Inhibitor of Early and Late Events in Herpes Simplex Virus Replication

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Unlike amantadine (1-adamantanamine), tromantadine (*N*-1-adamantyl-*N*-[2-(dimethyl amino)ethoxy]acetamide hydrochloride) inhibits herpes simplex virus type 1 (KOS strain)-induced cytopathic effect and virus replication with limited toxicity to the cells. Vero and HEp-2 cells tolerated up to 2 mg of tromantadine per 2×10^6 cells for 24-, 48-, or 96-h incubation periods with little change in cell morphology. Treatment of the cells with 10 to 50 μg of tromantadine reduced herpes simplex virus-induced cytopathic effect. Treatment with 100 to 500 μg of tromantadine inhibited herpes simplex virus-induced cytopathic effect and reduced virus production. Complete inhibition of virus production was observed with treatments of 500 μg to 1 mg. The antiherpetic activity of tromantadine was dependent upon the viral inoculum size and the time of addition of the compound with respect to infection. Virion synthesis and viral polypeptide synthesis were inhibited by addition of tromantadine at the time of infection or 4 h postinfection. The results are consistent with tromantadine inhibition of an early event in herpes simplex virus infection, before macromolecular synthesis, and a late event, such as assembly or release of virus.

Tromantadine (*N*-1-adamantyl-*N*-[2-(dimethylamino)ethoxy]acetamide hydrochloride) (10, 11, 16, 20) is one of relatively few amantadine (1-adamantanamine) derivatives which have antiherpetic activity. Amantadine has been shown to inhibit an early event before viral macromolecular synthesis in influenza A virus replication and is an alternative to vaccination against influenza A virus (15). Tromantadine has been reported to inhibit herpes simplex virus type 1 (HSV-1) and HSV-2 replication and to be effective as a topical antiherpetic drug (1, 4, 7, 9, 12, 16, 20, 25). In this paper, we report an improved synthetic scheme for the compound and describe its activity against HSV-1 infection. An indication of the HSV replication step(s) inhibited by tromantadine is discussed.

MATERIALS AND METHODS

Chemicals. Methylamine, amantadine, tetrahydrofuran (THF), *N,N*-dimethylethanolamine, and chloracetyl chloride were obtained from Aldrich Chemical Co. Acrylamide, diallyltartardiamide, dithiothreitol, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories. Epon 812, osmium tetroxide, uranyl acetate, and glutaraldehyde were obtained from Ernest Fullam, Inc., Schenectady, N.Y. Mixed ^{14}C-amino acids and [^{3}H]thymidine were obtained from ICN, Irvine, Calif.

Tromantadine synthesis. *N*-(1-Adamantyl)-2-chloroacetamide was synthesized as the precursor to tromantadine. For one preparation, 4.0 g (0.026 mol) of amantadine, 4.0 ml (0.28 mol) of triethylamine, and 25 ml of THF were stirred in a round-bottom flask cooled in an ice bath. A solution of 3.0 g (0.026 mol) of chloracetyl chloride in THF was added slowly. When the addition was complete, the ice bath was removed, and stirring was continued overnight. The contents of the flask were then poured into 5% HCl solution, and the resulting solution was extracted twice with 50-ml portions of diethyl ether. The combined ether extracts were washed with water and saturated NaCl solution and dried over anhydrous Na_2SO_4. Removal of the solvent gave 5.0 g (0.22 mol, 85%) of *N*-(1-adamantyl)-2-chloroacetamide (^{13}C nuclear magnetic resonance: δ 164.62 [*C*O], 52.39 [*C*H$_2$Cl], 42.93 [*C*—NH], 41.33 [β—*C*H$_2$], 36.30 [α—*C*H$_2$], 29.45 [bridgehead *C*H]).

Tromantadine was prepared by addition of *N,N*-dimethylethanolamine to *N*-(1-adamantyl)-2-chloroacetamide. First, the lithium alkoxide of *N,N*-dimethylethanolamine was prepared by the addition of 4.3 ml (0.009 mol) of 2.1 M *n*-butylithium in hexane to a cold solution of the amino alcohol (0.78 g, 0.0088 mol) in 10 ml of dry THF. A solution of *N*-(1-adamantyl)-2-chloroacetamide (2.0 g, 0.0088 mol) in 15 ml of dry THF was then added slowly, and the mixture was stirred overnight. The solution was next poured into 50 ml of 10% HCl, and the resulting solution was washed with ether. Addition of NaOH solution liberated the tromantadine, which was extracted into two 100-ml portions of ether. The combined ether extracts were dried over anhydrous Na_2SO_4, and the ether was evaporated to yield a viscous oil. The oil was dissolved in benzene, and tromantadine was precipitated by

TABLE 1. Dose response of HSV-1 replication to tromantadine[a]

Tromantadine (μg)[b]	Virus production (HEp-2) (PFU)		Virus production (Vero) (PFU)	
	24 h	72 h	48 h	72 h
0	4.2×10^3	2.6×10^8	2×10^8	4×10^8
0.1	—[c]	1.1×10^8	—	—
1.0	5.8×10^3	4×10^7	—	—
5	2.6×10^3	7.1×10^8	—	4×10^8
10	5.3×10^3	2.4×10^8	$>10^8$	$>10^8$
50	0	1.8×10^6	2.7×10^7	1.3×10^8
100	0	1×10^5	1.6×10^7	10^8
500	0	0	0	5×10^4
1,000	0	0	0	0

[a] The MOI was approximately 0.25.

[b] Tromantadine in 0.5 ml was added to cells 15 min before infection and was present throughout the 1-h infection period. After removal of the inoculum, the identical dose in 2.0 ml was reapplied to the cells.

[c] —, Not tested.

bubbling dry HCl gas. The precipitate was collected by suction filtration and washed with benzene. The compound is very hygroscopic. The yield was 2.0 g (0.0063 mol, 72%); the mp was 80 to 85°C. The ^{13}C nuclear magnetic resonance analysis of tromantadine was as follows: δ 169.00 (s, *C*O), 70.97 (t, $COCH_2O$), 69.79 (t, OCH_2), 58.94 (t, CH_2N), 51.46 (s, *C*—NH), 45.88 (q, $N—CH_3$). 41.52 (t, β-CH_2 of Ad), 36.41 (t, δ CH_2 of Ad) 29.48 (d, bridgehead *C*H of Ad). Letters are multiplicities in an off-resonance decoupled spectrum. Ad = Adamantyl.

Cells. HEp-2 and Vero cells were grown in 35-mm^2 plates (Costar) and maintained in Dulbecco minimal essential medium Auto-Pow medium (Flow Laboratories, Inc.) supplemented with newborn calf serum (15%) and antibiotics (penicillin G, 25 U/ml; streptomycin, 25 U/ml; L-glutamine, 29.2 μg; gentamicin, 10 μg/ml). Maintenance medium was supplemented with only 2% newborn calf serum. Tris-salts consisted of 10 mM Tris-hydrochloride (pH 7.4) and 150 mM NaCl.

Virus. HSV-1 (KOS strain) was provided by Priscilla Schaffer, Sidney Farber Cancer Institute, Boston, Mass. A single virus stock, prepared by infection of HEp-2 cells, was used for these experiments. Titers of infectious virus were determined by plaque assay on Vero cell monolayers after dilution in Tris-salts (6), and particle counts were determined by electron microscopy. The ratio of viral particles to plaque-forming units for this virus stock was ca. 9.

Determination of cytotoxicity and antiviral activity. Confluent monolayers of Vero (2.4×10^6 per plate) or HEp-2 (1.6×10^6 per plate) cells were treated with various amounts of tromantadine 15 min before and then throughout the infection and incubation. The dose of tromantadine applied, rather than the concentration, was varied since, as for amantadine (21), the cells concentrated the compound from the medium. For most assays, cells were infected with 6×10^5 PFU of HSV-1 in 0.5 ml for 1 h at 37°C, and the virus solution was then aspirated off the monolayer and replaced with 2 ml of maintenance medium containing the same amount of the compound.

The antiviral activity of the compounds was determined as a reduction in both virally induced cytopathic effect (CPE) and production of extracellular infectious virus 24, 48, and 96 h postinfection. Control cell monolayers could not be maintained past 96 h. In one assay, we determined total infectious virus content in the extracellular media and cell-free lysates of tromantadine-treated, HSV-1-infected HEp-2 cells. The lysates were prepared by freeze-thaw disruption of the cells in 0.1% EDTA–1 mM sodium phosphate (pH 7.4). The toxicities of tromantadine and amantadine were evaluated by determining their CPE on mock-infected cells and by determining [^3H]thymidine uptake. Cells treated with various doses of tromantadine and [^3H]thymidine were incubated for 48 h, washed once, solubilized with 1% (wt/wt) SDS, and scintillation counted. The effects of tromantadine on viral integrity and infectivity were tested by incubation of virus with the compound as described earlier for assays of antiherpetic activity in the absence of cells. After 1 or 24 h, viral infectivity of compound-treated virus, as measured by plaque assay, was compared with that of control-treated virus.

Time course of tromantadine action. Vero cells were treated with 1.0 mg of tromantadine in 2 ml at different times in the virus replicative cycle. Tromantadine was present 15 min before infection and then removed, 15 minutes before and then throughout infection, 1 h during infection only and then removed, 1 h at 4 h postinfection only, or 4 h postinfection to the end of the experiment. Infection of the monolayers was carried out as described above. When appropriate, cells were washed three times with Tris-salts to remove the compound before replacement with medium.

HSV polypeptides were assayed in terms of time of tromantadine addition relative to infection. Tromantadine and mixed ^{14}C-amino acids were added either 15 min before infection or 4 h postinfection (multiplicity of infection [MOI], <1), as described above. After 24 h, the cells were washed, scraped off the dish, pelleted, washed twice, and lysed by boiling in SDS sample buffer with 0.1 M dithiothreitol. Cell lysates were electrophoresed on 8.6% diallyltartardiamide-cross-linked SDS-polyacrylamide gels (17), and the polypeptides were visualized by fluorography (3).

Electron microscopy was performed on cells which were mock infected, infected with HSV, infected with HSV and treated with 1 mg of tromantadine 15 min before and throughout HSV infection and incubation, or infected and treated with tromantadine 4 h postinfection. After 24 h, the cells were fixed in 1.25% glutaraldehyde, postfixed in 1% osmium tetroxide, and

TABLE 2. Effect of increasing virus concentration on tromantadine activity

Input virus (PFU × 10^5)	Virus production (PFU)[a]			
	24 h		48 h	
	With tromantadine[b]	Without tromantadine (×10^6)	With tromantadine[b]	Without tromantadine (×10^6)
3	0	2	0	3
6	0	19	0	18
12	0	48	0	42
18	10^4	18	5×10^4	54
24	10^4	27	9×10^4	41
60	2×10^4	84	17×10^4	75
90	4×10^4	88	11×10^4	103

[a] Total extracellular virus.
[b] Dose of 1 mg of tromantadine per plate.

embedded in Epon 812 (18), all in situ. Thin sections of the embedded cells were stained with uranyl acetate and viewed with a JEOL 100-S electron microscope.

RESULTS

Dose-response studies. Before we tested the antiherpetic activity of tromantadine and amantadine, we evaluated the toxicity of the compounds to HEp-2 or Vero cells. The solubility of tromantadine in aqueous buffers at physiological pH allowed assay of its activity by direct addition to culture medium. Addition of up to 2 mg of tromantadine in 2 ml had little effect on the morphology of either HEp-2 or Vero cells over a 96-h period. Plating efficiency and outgrowth of HEp-2 cells, as well as [^{3}H]thymidine and ^{14}C-amino acid uptake by subconfluent cell monolayers, were equivalent to control levels at tromantadine doses of at least 1.2 mg. Tromantadine doses higher than 2 mg induced vacuolation of cells and subsequent lysis regardless of the volume of administration. Amantadine at doses of 2 mg or more, also induced vacuolation of the cytoplasm and lysis of both cell types within 24 h of treatment. The dose dependence of autolysis, rather than the concentration dependence, suggests that these compounds are sequestered from the medium by the cells.

Inhibition of HSV-induced CPE, which is characterized by cell rounding and lysis and monolayer disruption, was used as the criterion of an initial assay for antiherpetic activity. HEp-2 or Vero cells treated with 100 μg of tromantadine for 15 min before and then throughout infection and incubation exhibited no CPE for 96 h. Treatment with 10 to 50 μg of tromantadine reduced the extent of HSV-induced CPE.

The activity of tromantadine was also evaluated by determining inhibition of virus production. This was quantified by plaque assay of samples of cell-free medium taken from tromantadine-treated, HSV-infected cells at 24, 48, and 96 h postinfection. Tromantadine treatments of 100 to 500 μg inhibited the production of virus 24 h postinfection, sufficient time for the initial round of virus replication. However, virus production could be observed after 48 h upon treatment with less than 500 μg. Vero cells gave similar results under these assay conditions; however, a higher concentration of tromantadine was required for inhibition of virus replication (Table 1). Amantadine had no effect on virus production (data not shown).

In one experiment, HEp-2 cells were treated with 1 mg of tromantadine, infected with HSV-1, and incubated at 34°C for 4 days. No CPE or virus production was observed. After the 4-day incubation period, the cells were washed free of the compound and incubated for 2 more days. No subsequent virus production or change in cellular morphology was observed.

The effect of tromantadine on the integrity of HSV was evaluated by assaying for infectivity after a 1- or 24-h incubation with tromantadine. After the incubation period, virus and tromantadine were diluted at least 10^4-fold before plaque assay. No significant loss of infectivity was observed upon incubation with doses of tromantadine of 0.2 to 1.2 mg for either time period.

To determine whether virus replication, rather than release of virus from the cells, was being inhibited, we assayed for intracellular as well as extracellular virus. Cells treated with 1 mg of tromantadine and HSV were incubated for 24 h, and the monolayer was then washed and lysed by hypotonic shock on 0.1% EDTA and freeze-thawing. No virus could be detected in either the cell lysate or the medium of the tromantadine-treated cells, whereas 2×10^5 PFU of intracellular virus and 1.2×10^5 PFU of extracellular virus were detected in untreated cells. Thus, the decrease in extracellular virus after tromantadine treatment indicated a reduction in total virus yield.

TABLE 3. Time course of tromantadine action[a]

Time of tromantadine addition	Treatment period[b]	24 h		48 h	
		Virus titer	% Inhibition	Virus titer	% Inhibition
No tromantadine		2.16×10^6		7×10^7	
Present throughout[c]	−0.25–48 h	3.8×10^3	99.9	7.4×10^4	99.9
At time of infection[c]	0–48 h	2.6×10^3	99.9	5.5×10^4	99.9
15 min before infection, then removed[d]	−0.25–0	6.4×10^5	70	1.8×10^8	0
During infection, then removed[d]	0–1 h	1.8×10^4	99.2	1.65×10^7	0
4 h postinfection	4–48 h	1.58×10^4	99.3	1.2×10^5	99.8

[a] Dose of tromantadine was 1 mg. See text for experimental details.
[b] Time relative to infection.
[c] After the 1-h incubation period, the inoculum was removed, and 1 mg of tromantadine in 2 ml of medium was reapplied to the cells without washing of monolayer. Medium taken from cells immediately postinfection and posttreatment contained 4×10^3 virus PFU per 0.1 ml.
[d] The cells were washed three times with Tris-salts to remove compound.

Relationship between virus concentration and tromantadine activity. The effect of viral inoculum size on the efficacy of tromantadine treatment was studied as described above. HEp-2 cells were treated with 1 mg of tromantadine and infected with between 3×10^5 and 9×10^6 PFU of HSV. Virus production was then assayed 24 and 48 h postinfection. The viral inoculum size used for the previous experiments was 6×10^5 PFU per plate. Tromantadine inhibition of HSV replication was dependent upon viral inoculum size (Table 2). However, at all virus concentrations used, tromantadine reduced the level of virus production by more than 99%. Complete inhibition of virus production by tromantadine was observed for viral inocula of 12×10^5 PFU or less.

Effect of time of tromantadine addition. Variation of the time of tromantadine addition relative to infection was used to indicate the step in virus replication inhibited by tromantadine (14). Vero cells were treated with 1 mg of tromantadine throughout infection and incubation (at least 24 h), 15 min before infection only, during the 1-h infection period only, for 1 h at 4 h postinfection only, or 4 h postinfection to the end of the experiment. Infection was then evaluated by plaque assay, determination of virion polypeptide synthesis, and electron microscopy. In a normal infection, HSV α- and β-polypeptide synthesis is initiated and cellular protein synthesis is inhibited within 4 h (8).

In Table 3, the effect of time of tromantadine addition on infectious virus production is shown. Whereas untreated cells produced 2×10^6 PFU of virus within 24 h, Vero cells treated with tromantadine as described above produced only 3×10^3 PFU. Tromantadine treatment of cells for 15 min before infection or for a 1-h period at 4 h postinfection did not significantly affect HSV replication. However, treatment of cells during the 1-h infection period inhibited the production of virus for one round of virus replication (24 h) by more than 99%. Removal of tromantadine after the 1-h treatment allowed subsequent rounds of virus replication to occur, as determined 48 h postinfection. Addition of tromantadine 4 h postinfection also inhibited virus production by more than 99%. The reduction in virus production was still observed 48 h postinfection. These results suggest that the compound inhibits virus replication both early and late in infection, or less likely, that the compound is sequestered by cells early in infection but inhibits a later step in virus replication.

The effect of tromantadine on HSV polypeptide synthesis was studied by treating HEp-2 cells with tromantadine and ^{14}C-amino acids at various times relative to HSV infection. Viral polypeptide synthesis 24 h postinfection could be detected by the presence of a viral polypeptide(s) of approximately 122,000 daltons (Fig. 1, arrow) against the background of cellular proteins. Addition of tromantadine before infection inhibited synthesis of at least this viral polypeptide. (Fig. 1). Addition of tromantadine 4 h postinfection did not inhibit further production of this viral polypeptide (Fig. 1).

Electron micrographs of HSV-infected cells treated with 1 mg of tromantadine at infection or 4 h postinfection were also compared with micrographs of uninfected and infected controls. HSV-infected HEp-2 cells contained intracellular and intranuclear virus with visible HSV capsid paracrystals in the nucleus. Cells treated with tromantadine at the time of infection showed no intracellular or intranuclear evidence of virus infection. Virus could, however, be observed bound to or associated with the outside of the cell. Viral capsids and electron-dense granules in the nucleus were observed in cells treated with tromantadine at 4 h postinfection.

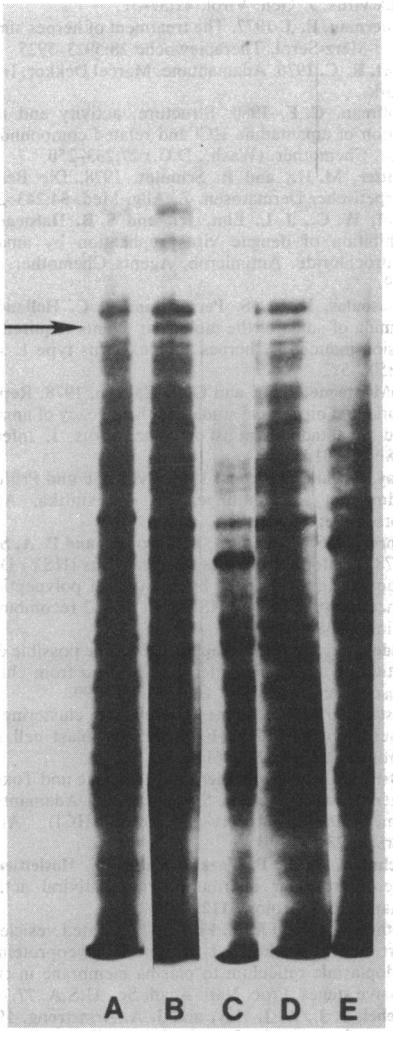

FIG. 1. SDS-polyacrylamide gel electrophoresis of control and tromantadine-treated HSV-infected HEp-2 cells. HEp-2 cells were untreated and uninfected (A), infected with an MOI of 0.25 (B), infected with HSV and treated with 1 mg of tromantadine 15 min before and throughout infection and incubation (C), infected with HSV and treated with 1 mg of tromantadine 4 h after infection (D), or uninfected and treated with 1 mg of tromantadine (E). Cells were labeled with ^{14}C-amino acids 1 h postinfection and harvested 24 h after infection. Samples were prepared for electrophoresis and fluorography as described in the text. Arrow, Location of 122,000-dalton viral polypeptide(s).

However, the majority of viral capsids lacked an electron-dense core and did not exhibit the typical HSV capsid morphology.

DISCUSSION

Tromantadine is a derivative of amantadine, but unlike the parent compound, tromantadine is an effective inhibitor of HSV replication. The work described above was stimulated by the clinical and research potential of tromantadine and by an insufficient amount of background information published about the compound. After attempting to reproduce the synthetic protocol described by May and Peteri (16), we found it necessary to develop a new method for tromantadine production. This new procedure is easier, more reproducible, produces greater yields, and is more adaptable than the other procedure. We have extended the previously reported studies to quantitatively evaluate the activity of the compound on a defined tissue culture system of a well characterized HSV-1 strain, KOS. This enables standardization of assays of antiherpetic activity for future studies on the mode of action of the compound.

Tromantadine inhibits HSV-1 KOS strain replication and virus-induced CPE at doses well below toxic concentrations and within the concentration range reported for amantadine inhibition of influenza A virus (23), lymphocytic choriomeningitis virus (24), or dengue virus (13). Doses of at least 100 μg of the compound reduced virus production and CPE, whereas higher doses prevented virus replication. The compound inhibited HSV replication in two tissue culture cell types. The activity was dependent on the infectious inoculum size, but the effect was not mediated by direct destruction of virus.

Amantadine has been shown to inhibit an early event in influenza A virus replication, the uncoating of the virus. Amantadine-resistant mutants have an altered M protein, indicating a role for this protein in the anti-influenza virus activity of the compound (2).

The chemical properties of tromantadine and its structural similarities to amantadine suggest similar modes of action for the two compounds. Like amantadine, tromantadine is concentrated by cells and can initiate autolysis at high doses. Amantadine is rapidly concentrated in the lysosomes and the cytosol of MDCK cells, but most of the cell-bound amantadine is unavailable for antiviral activity (21). The activities of both amantadine and tromantadine are dependent upon the inoculum size of the respective target virus (influenza [11] and HSV-1 [Table 2]). In addition, relatively large amounts of both compounds are required for inhibition of virus replication (11; Tables 1 and 2).

Unlike amantadine, however, tromantadine inhibits HSV replication. Examination of other amantadine derivatives indicates the importance of an amide or thioamide group for antiherpetic activity, whereas for tromantadine, the side chain potentiates that activity (10, 11, 16, 20). Weak bases other than tromantadine, such as

NH_4Cl, butylamine, and chloroquine, have been shown to have lysosomatropic activity (5) and can also disrupt coated vesicles (19, 22), cellular structures important for maturation of some viruses (22). Inhibition by tromantadine of both an early event and a late event in HSV replication suggests that tromantadine inhibits an early event in HSV replication, as does amantadine for influenza A virus, and is also capable of inhibition of a cellular function required for maturation of viral polypeptides or virion assembly.

Our results confirm that tromantadine is effective against HSV-1 replication and indicate the step in virus replication inhibited by the compound. In addition to determining the mode of action of tromantadine, future studies will utilize this compound as a tool to dissect and define the early events in HSV replication.

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